SUPEROXIDE DISMUTASE AS A VACCINE ANTIGEN

Amd. C1

The present invention relates to pharmaceutical compositions for treating and/or vaccinating against bacterial infection and to methods of manufacturing such compositions. In particular, the invention relates to pharmaceutical compositions which comprise superoxide dismutase or antibodies thereto.

At present most bacterial infections in humans or animals are treated after infection has set in by administration of antibiotic drugs. As many more strains of pathogenic bacteria become resistant to current antibiotics, the range of options open for treatment decreases. Moreover, many antibiotics can cause dangerous side effects upon individuals or animals taking them, for example allergy to penicillin or the toxicity of sulpha drugs. Furthermore, antibiotic treatment is sometimes only effective if the drug is taken regularly over a period of time thus maintaining a constant level of therapeutic agent in circulation. If individuals forget or are unable to maintain the course of antibiotic treatment then it may be rendered ineffective.

Some bacterial infections progress very quickly, sometimes too quickly for antibiotic treatment to have much effect unless administered at a very early stage in the course of the infection. Meningococcal disease is one example of such a virulent infection and is caused by the pathogen *Neisseria meningitidis*. In many cases symptoms of disease at first resemble those of influenza and thus infected individuals often delay in seeking medical attention. Vaccines based on polysaccharides present on the surface of some *N. meningitidis* serogroups are available at present but they show limited protection against infection. Moreover, the surface polysaccharide of serogroup B strains of *N. meningitidis* (causative agent of over half the cases of meningococcal disease in the UK) are only weakly immunogenic and are not included in current vaccines.

Accordingly, it is an object of the invention to provide a pharmaceutical composition comprising a vaccine antigen or an antibody that effectively protects against or ameliorates bacterial infection. It is a further object of the invention to provide a pharmaceutical composition comprising a vaccine antigen that protects against meningococcal disease. It is yet a further object to provide a vaccine antigen that also provides protective immunity against a broader range of infectious bacteria. It is a further object to provide a method of manufacturing antibodies that can provide protective immunity to a range of bacterial pathogens when included in a pharmaceutical preparation. It is still a further object of the invention to provide a multivalent vaccine which provides protective immunity to a wide range of bacterial infections.

Cu,Zn-Superoxide Dismutase (Cu,Zn-SOD) is an metalloenzyme found in many prokaryotic and eukaryotic organisms. It catalyses the reduction of the superoxide radical anion, O_2^-, to hydrogen peroxide and molecular oxygen, thus playing an important role in the removal of cytotoxic free radicals from the organism. In bacteria Cu,Zn-SODs have been identified in the periplasm of a number of Gram negative species including *N. meningitidis*, *Haemophilus ducreyi*, *Haemophilus parainfluenzae*, *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* (Kroll et al, 1995). The enzyme can exist as a dimer or a monomer, and examples of monomeric Cu,Zn-SODS are those from *Brucella abortus* and *Escherichia coli* (Pesce, et al, 1997). It is believed that Cu,Zn-SOD provides a defence for the bacterium against the burst of oxygen free radicals released by phagocytic host cells, such as macrophages, during infection (Wilks et al, 1998; Farrant et al, 1997).

A known attempt to utilise Cu,Zn-SOD as a vaccine antigen has not been successful. Tabatabai (Tabatabai and Pugh, 1994) showed that a synthetic fragment of a monomeric Cu,Zn-SOD (denoted as peptide 3) from *B. abortus* was able to provide a low level of immunity in mice against *Brucella* infection, but the level of protection provided was lower than that seen when using

Brucella cell surface proteins and lipopolysaccharide antigens. Moreover, vaccination with the entire Cu,Zn-SOD failed to provide protective immunity at all. Tabatabai concluded that the antigenic fragment contained within *Brucella* Cu,Zn-SOD was counteracted by other parts of the protein which prevented it from eliciting an antigenic activity. This masking property was in fact so strong that even a mixture of synthetic peptides that included peptide 3 elicited no protective immunity to *Brucella* infection. Thus the study by Tabatabai et al teaches against the use of either fragments of Cu,Zn-SOD or full length Cu,Zn-SOD as an effective antigen that could provide protective immunity to bacterial infection.

Cu,Zn-SODs from eukaryotes and most Gram negative bacteria form dimers in their native form. However, the Cu,Zn-SODs of *E.coli* and *B. abortus* are atypical in that they are normally monomeric (Pesce et al, 1997). In all Gram negative bacteria that produce Cu,Zn-SOD the protein is localised to the periplasmic space between the outer cell wall and the cell membrane.

The present invention relates to the surprising discovery that a monoclonal antibody raised against a recombinant Cu,Zn-SOD fusion protein from *A. pleuropneumoniae* has bactericidal activity against *N. meningitidis* and protects mice against challenge with *N. meningitidis*. Furthermore, a recombinant, dimeric Cu,Zn-SOD and immunogenic fragments from *A. pleuropneumoniae* (a pig pathogen) act as antigen that can confer protective immunity not only against this organism but also against other Gram negative human pathogens such as *N. meningitidis*, *H. ducreyi*, *H. parainfluenzae* and *P. multocida*. Thus the Cu,Zn-SOD is suitable as a vaccine component against both animal and human bacterial diseases such as meningococcal disease (caused by *N. meningitidis*) or chancroid (caused by *H. ducreyi*), or porcine pleuropneumonia (caused by *A. pleuropneumoniae*).

Accordingly, a first aspect of the present invention provides a composition comprising a Cu,Zn-SOD of the dimeric type, or a fragment, variant or

derivative thereof, and a pharmaceutically acceptable carrier. The first aspect of the invention also provides a composition comprising a nucleic acid encoding a Cu,Zn-SOD of the dimeric type, or a fragment, variant or derivative thereof, and a pharmaceutically acceptable carrier. By "dimeric" we mean a SOD that naturally forms dimers under normal conditions, e.g. is found in dimeric form in nature. A pharmaceutically acceptable carrier could comprise an approved adjuvant such as alum or any other adjuvant approved for pharmaceutical purposes. The Cu,Zn-SOD can be from any bacteria, though especially from known pathogenic bacteria, and from *N. meningitidis* as an example.

The present invention is not to be restricted to the use of full length or wild type Cu,Zn-SOD of the dimeric type. An antigenic fragment of the Cu,Zn-SOD may also be used in a vaccine formulation. The fragment preferably comprises a region of the Cu,Zn-SOD that is on the surface of the protein, although any fragment that confers protective immunity to bacterial infection is suitable; and the term "fragment" is intended to encompass any fragment against which an antibody may be raised which antibody binds intact, full length SOD. Moreover, mutant variants which have been modified to increase antigenicity or fusion protein derivatives between all or a part of a Cu,Zn-SOD and another protein for the purposes of purification or increasing antigenicity may also be suitable for use in pharmaceutical compositions. Vaccine components of the invention also include derivatives and variants of Cu,Zn-SOD. The term "derivative" is intended to encompass combinations of Cu,Zn-SOD with other proteins or molecules, including carbohydrates to form conjugate vaccines, the derivative retaining antigenicity such that an antibody raised against the derivative binds intact, full length SOD. The term "variant" is intended to encompass a polypeptide having an amino acid sequence that varies from that of intact, full length SOD, but such that antibodies raised against the variant bind intact, full length SOD.

In a preferred embodiment of the invention the pharmaceutical composition

provides protection against meningococcal infection and/or disease. In further preferred embodiments of the invention the pharmaceutical composition provides protective immunity to infection from *Actinobacillus* species (e.g. *A. pleuropneumoniae, A. actinomycetemcomitans*), *Pasteurellaceae* species (e.g. *P. multocida*), *Neisseria* species (e.g. *N. meningitidis*), *Haemophilus* species (e.g. *H. influenzae, H. parainfluenzae, H. ducreyi*), *Escherichia coli*, *Salmonella* species and other bacteria producing a Cu,Zn-SOD. In a specific embodiment of the invention the Cu,Zn-SOD is expressed from a recombinant gene cloned from *Actinobacillus pleuropneumoniae*.

It is an advantage of the present invention that a Cu,Zn-SOD of the dimeric type, or a fragment, variant or derivative thereof, can confer protective immunity to infection from a broad range of bacterial pathogens. It is of further advantage that the present invention provides for pharmaceutical compositions comprising Cu,Zn-SODs, or a fragment, variant or derivative thereof, that are protective against bacterial infection in both humans and animals and in particular to meningococcal disease. It is of still further advantage that an antibody to a Cu,Zn-SOD from one species of bacteria can provide protective immunity to infection from a plurality of other species of bacteria, in particular that the invention provides protective immunity to meningococcal disease. A further advantage of the present invention is that Cu,Zn-SOD is relatively abundant and can be easily purified from bacterial cultures. Moreover, recombinant Cu,Zn-SODs can be fused to other proteins, such as glutathione-S-transferases, to facilitate purification from bacterial cultures expressing the fusion protein, and the Cu,Zn-SOD moiety retains both antigenicity and biological activity.

In use of the invention, a Cu,Zn-SOD is cloned from the pig pathogen *Actinobacillus pleuropneumoniae*, to give a recombinant form of the gene. The recombinant Cu,Zn-SOD gene is optionally linked to, such as by fusing, a glutathione-S-transferase gene to enable easy purification of the fusion protein when expressed in bacteria. A pharmaceutical preparation is prepared

comprising the purified Cu,Zn-SOD protein and a pharmaceutically acceptable carrier; in one use the carrier includes the adjuvant alum. The pharmaceutical composition is suitably administered to the individual via any route. The nature or form of the composition may be selected from any conventional pharmaceutical composition including but not limited to tablets, capsules, oral compositions, liquids, compositions for infusion, syrups, solutions, powdered formulations and granular formulations. The pharmaceutical composition, in use, stimulates the individual to produce antibodies against the antigenic Cu,Zn-SOD protein, some of which provide protective immunity to a broad range of pathogens. In particular the pharmaceutical composition provides protective immunity to meningococcal disease.

In a specific example of the invention in use, described in more detail below, a Cu,Zn-SOD gene - the *sodC* gene - is isolated from *N. meningitidis* genomic DNA by standard PCR techniques. The product of the PCR reaction additionally incorporates a His-tag sequence (coding for six histidines at the C terminus of the protein). The isolated *sodC* gene plus His-tag is cloned into an expression vector and then transformed into *E. coli*, where the protein product is expressed. The expressed protein is purified on a nickel charged affinity column, to which the His-tag preferentially binds. The SodC protein is then eluted from the affinity column and is suitably incorporated in the pharmaceutical composition of the invention as described above.

A second aspect of the invention provides a vaccine comprising a Cu,Zn-SOD, or a nucleic acid encoding a Cu,Zn-SOD, of the dimeric type, or a fragment, variant or derivative thereof.

In a specific embodiment of the invention the Cu,Zn-SOD of the dimeric type, or a fragment, variant or derivative thereof, is from a recombinant gene cloned from *Actinobacillus pleuropneumoniae*, though the invention also encompasses use of native proteins. In a further preferred embodiment the vaccine provides protective immunity against meningococcal meningitis.

Compositions and vaccines of the invention comprising a nucleic acid which encodes a Cu,Zn-SOD or fragment or derivative thereof are suitably prepared comprising the coding sequence inside a microparticle according to the methods of WO-A-97/17063, incorporated herein by reference.

A third aspect of the invention provides a method of preparing a pharmaceutical composition that consists of:-

1) cloning a gene for a Cu,Zn-SOD of the dimeric type to obtain a recombinant form of the gene; and

2) (a) synthesising Cu,Zn-SOD from the recombinant gene; and combining said Cu,Zn-SOD with a pharmaceutically acceptable carrier, or

(b) combining said gene with a pharmaceutically acceptable carrier.

A fourth aspect of the invention provides for a composition, especially a pharmaceutical preparation comprising an antibody to a Cu,Zn-SOD of the dimeric type, or a fragment, derivative or variant thereof, and a pharmaceutically acceptable carrier. It is optional, but not essential, that the antibody is a monoclonal antibody.

The present invention thus also provides for an antibody preparation that is raised against a dimeric Cu,Zn-SOD, or a fragment, derivative or variant thereof, from one species of Gram negative bacteria and that confers protective immunity to infection from this bacterium and also to infection from a plurality of other Gram negative bacteria. The antibody can be used in pharmaceutical preparations that confer passive immunity to bacterial infection upon a host organism. In the example described in more detail below a monoclonal antibody raised against Cu,Zn-SOD from *A. pleuropneumoniae* provides protection against *N. meningitidis* infection. Thus

the monoclonal antibody is suitable for use in treating acute cases of meningococcal disease as well as in providing passive immunity to future meningococcal disease.

In a specific embodiment of the invention the antibody provides protective immunity to meningococcal disease. Thus it is suitable for use in preparations that provide passive immunity to infection and also for treatment of individuals already suffering from meningococcal infection.

In further specific embodiments of the invention the antibody provides protective immunity to bacterial infection from a range of bacterial pathogens including *Actinobacillus pleuropneumoniae*, *Pasteurellaceae* species, *Neisseria* species and *Haemophilus* species.

In a further preferred embodiment of the invention the antibody displays bactericidal activity. This activity may be directly attributed to the antibody itself or mediated via the complement system.

A fifth aspect of the invention provides for a multivalent vaccine comprising a plurality of Cu,Zn-SODs of the dimeric type, or fragments, derivatives or variants thereof, wherein said plurality of Cu,Zn-SODs are from the same or different species of Gram negative bacteria. A specific embodiment of the invention provides a multivalent vaccine comprising a plurality of Cu,Zn-SODs of the dimeric type, or fragments, derivatives or variants thereof, and one or a number of different bacterial proteins, or a fragment, derivative or variant thereof, that is not or are not a Cu,Zn-SOD. A further preferred embodiment of the invention is a multivalent vaccine that provides protective immunity to meningococcal infection.

A sixth aspect of the invention provides for use of a Cu,Zn-superoxide dismutase of the dimeric type, or a fragment, derivative or variant thereof, in the manufacture of a medicament, e.g. a vaccine, for use against bacterial

infection. In a preferred embodiment of the invention the use is in response to a bacterial infection due to Gram negative species of bacteria. In a specific embodiment of the invention the use is in response to a meningococcal infection. This aspect also provides a method of treating bacterial, particularly meningococcal, infection by administering an effective amount of a Cu,Zn-SOD or a fragment, variant or derivative thereof, or other composition according to the invention.

A seventh aspect of the invention provides for an antibody specific to bacterial Cu,Zn-SOD of the dimeric type, or a fragment, derivative or variant thereof. A preferred embodiment of the invention provides for a monoclonal antibody (MAb) that is specific to bacterial Cu,Zn-SOD.

There now follow examples of specific embodiments of the invention.

Example 1

Cu,Zn-SOD Monoclonal Antibody

Monoclonal antibodies (MAbs) were raised in mice against a glutathione-S-transferase fusion protein with the Cu,Zn-SOD from *Actinobacillus pleuropneumoniae*. The whole fusion protein as expressed in *E. coli* was found to be enzymically active. Of 72 potential hybridomas screened by western blotting, two recognised only *A. pleuropneumoniae* Cu,Zn-SOD, but one also recognised Cu,Zn-SOD *from H. parainfluenzae, H. ducreyi, N. meningitidis* and *A. actinomycetemcomitans*. The latter MAb was used for the passive protection studies.

Passive Protection

Three experiments were performed and all demonstrated that the MAb protects mice against lethal infections with *N. meningitidis.* Adult NIH mice were given an intra-peritoneal (ip) injection with antibody (50μl) per mouse 2h before challenge with *Neisseria meningitidis*. Mice were given further ip

injections of antibody 2,5 and 24h after the challenge. The *N. meningitidis* challenge dose was the stated number of viable bacteria (see tables of results), given as an 0.5ml ip injection, containing 2mg iron dextran. At 24h mice were given a further 2mg iron dextran in an 0.2ml ip dose. Mice were then observed for 72h and the health of the animals noted. The results of these experiments are shown in the following tables.

EXPERIMENT 1				
VACCINE	CHALLENGE DOSE	SURVIVORS / CHALLENGED		
		DAY 1	DAY 2	DAY 3
growth medium	10^5	5/5	4/5	4/5
growth medium	10^6	5/5	3/5	3/5
50μl MAb	10^5	5/5	5/5	5/5
50μl MAb	10^6	5/5	5/5	5/5
5μl MAb	10^5	5/5	5/5	5/5
5μl MAb	10^6	5/5	4/5	4/5
Polyclonal serum *	10^5	5/5	5/5	5/5
Polyclonal serum *	10^6	5/5	5/5	5/5

* - raised against meningococcal outer membrane vesicles.

EXPERIMENT 2				
VACCINE	CHALLENGE DOSE	SURVIVORS/CHALLENGED		
		DAY 1	DAY 2	DAY 3
Growth medium	2×10^5	5/5	5/5	5/5
Growth medium	2×10^6	5/5	1/5	0/5
50µl MAb	2×10^5	5/5	5/5	5/5
50µl MAb	2×10^6	5/5	5/5	5/5
5µl MAb	2×10^5	5/5	5/5	5/5
5µl MAb	2×10^6	5/5	3/5	3/5
Polyclonal serum *	2×10^5	5/5	5/5	5/5
Polyclonal serum *	2×10^6	5/5	5/5	5/5

* - raised against meningococcal outer membrane vesicles.

EXPERIMENT 3					
VACCINE	CHALLENGE DOSE	SURVIVORS / CHALLENGED			
		DAY 1	DAY 2	DAY 3	DAY 4
Growth medium	1×10^7	5/5	0/5	0/5	0/5
50µl MAb	1×10^7	5/5	4/5	3/5	2/5
5µl MAb	1×10^7	5/5	1/5	0/5	0/5
Polyclonal serum	1×10^7	5/5	1/5	0/5	0/5
50µl Yersinia MAb	1×10^7	5/5	1/5	0/5	0/5
5µl Yersinia MAb	1×10^7	5/5	0/5	0/5	0/5

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Bactericidal Activity

In addition to passive protective activity, the Cu,Zn-SOD MAb has been found to be bactericidal to two strains of meningococcus, with titres of 64-128 compared to a titre of 256 for a Group B polysaccharide specific MAb.

Example 2

Cloning and Expression of Cu,Zn-SOD - Purification of protein via a nickel affinity column

Genomic DNA isolated from *N.meningitidis* strain MC58 was used as a template to amplify the *sodC* gene (Cu,Zn-SOD). Primers were designed using the information in the Genbank database. The 5' primer (SEQ ID NO.1)

incorporates an *Nde*I site at the level of the ATG start codon, allowing the PCR product to be cloned into a variety of pET (Novagen) and pMTL (CAMR) vectors for expression. The 3' primer (SEQ ID NO.2) generates a polyhistidine (6 x His) tag in the translated protein to facilitate nickel affinity purification. The primer sequences are as follows:

"SEQ ID NO.1" : 5' primer: 5' GGC ATA TGA ATA TGA AAA CCT TAT TAG 3'

"SEQ ID NO.2" : 3' primer: 5' GGG CTG AGC TTA TTA GTG GTG GTG GTG GTG GTG TTT AAT CAC GCC ACA TGC CAT ACG TG 3'

The products were sub-cloned into pET22b and transferred to BL21 DE3 for initial expression. His-tagged protein was purified on a nickel charged Hi-Trap column.

Example 3

Purification of protein via a glutathione affinity column

A glutathione-S-transferase (GST) fusion was generated by cloning the *sodC* gene of Example 2 (described above) into one of the pGEX series of vectors (Pharmacia). An N-terminal fusion was generated by cloning a *sodC* PCR product as an *Eco*RI-*Xho*I fragment in frame into pGEX-4T-1 (restriction sites generated by PCR primers containing these sites in-frame immediately upstream or downstream of the *sodC* coding sequence, including 3' stop codon). The expressed protein was a GST-SodC fusion linked by a thrombin cleavage site. The recombinant protein was purified on a glutathione affinity column and cleaved with thrombin to release SodC. This SodC protein has an additional five amino acids on the N terminus, compared to wild type protein (sequence GSPQF).

Example 4

Purification of protein via a glutathione affinity column - Alternative method

This alternative cloning strategy varies from the method of Example 3 in that it allows reduction in the number of additional N terminal amino acids on the cleaved SodC product. This is achieved by removing the internal MC58 *sodC* *Bam*HI restriction site by site directed mutagenesis. PCR amplification of this altered gene with a 5' *Bam*HI site in-frame upstream of the ATG start codon facilitates cloning of a *Bam*HI-*Eco*RI fragment into pGEX-2T vector (Pharmacia). The resultant GST-SodC fusion protein is expressed and SodC released by cleavage with thrombin protease, giving rise to SodC product with just a two amino acid N terminal 'tag' (sequence GS).

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